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1 **Effective CO<sub>2</sub> capture by the fed-batch culture of *Chlorella vulgaris***

2

3 **Su Fang Li<sup>a</sup>, Chien Chih Wang<sup>b</sup>, Behnam Taidi<sup>a,b,\*</sup>**

4 <sup>a</sup>Laboratoire Génie des Procédés et Matériaux (LGPM), CentraleSupélec, Université Paris-Saclay, 91190

5 Gif-sur-Yvette, France

6 <sup>b</sup>Université Paris-Saclay, CentraleSupélec, Laboratoire Génie des Procédés et Matériaux, Centre

7 Européen de Biotechnologie et de Bioéconomie (CEBB), 3 rue des Rouges Terres 51110 Pomacle, France

8

9

10 \* *Corresponding author*

11 *E-mail address: behnam.taidi@centralesupelec.fr*

12

## 1 Abstract

2 Microalgae are promising candidates for CO<sub>2</sub> capture and concomitant biomass production. *Chlorella*  
3 *vulgaris* was grown in the batch and fed-batch cultures to develop a culture strategy targeting maximum  
4 CO<sub>2</sub> capture and conversion to biomass. Growth at five different dissolved CO<sub>2</sub> (dCO<sub>2</sub>) concentrations in  
5 the feeding media (atmospheric, 1.55, 1.62, 1.75 and 1.88 g L<sup>-1</sup>) was tested to assess the effect of dCO<sub>2</sub>  
6 concentration on the growth, biomass productivity, and nutrients removal efficiency of microalgae. Results  
7 suggest that fed-batch culture outperformed “standard” batch cultivation with a higher algal growth rate (2.3  
8 times). As expected, the algal growth was limited at low (atmospheric) dCO<sub>2</sub> concentrations and inhibited  
9 at high levels (1.75 and 1.88 g L<sup>-1</sup>). *C. vulgaris* grown with medium containing 1.62 g L<sup>-1</sup> dCO<sub>2</sub> showed the  
10 highest growth rate (0.094 h<sup>-1</sup>) with the shortest doubling time (7.4 h), maximum biomass productivity,  
11 nitrogen and phosphorus uptake rates (222, 7.5 and 1.6 mg L<sup>-1</sup> d<sup>-1</sup>, respectively). By pre-dissolving CO<sub>2</sub> into  
12 the feeding media and using the fed-batch culture mode, a high CO<sub>2</sub> removal efficiency could be achieved.

13  
14 **Keywords:** Batch cultivation, Biomass productivity, *Chlorella vulgaris*, CO<sub>2</sub> capture efficiency, Fed-batch  
15 cultivation, Nutrients removal

## 17 1. Introduction

18  
19 CO<sub>2</sub> emission due to fossil fuel combustion nowadays is one of the greatest environmental concerns. To  
20 mitigate the global greenhouse process, CO<sub>2</sub> capture has become an urgent issue. Photosynthetic organisms,  
21 such as plants and algae, are candidates for sequestering CO<sub>2</sub> via photosynthesis with the added possibility  
22 of economically converting CO<sub>2</sub> into high-value biomolecules: polysaccharides, proteins, lipids, pigments,  
23 etc. [1]. Compared to higher plants, microalgae-based CO<sub>2</sub> capture appears to have greater prospects because  
24 of the higher photosynthetic and CO<sub>2</sub>-uptake efficiencies (10 – 50 times) [2–4] due to the rapid growth cycle

1 of these microorganisms. Microalgae possess the remarkable potential to sequester and convert 513 tons of  
2 CO<sub>2</sub> into 280 tons of dry biomass per hectare per year [5], surpassing the peak fixation rate of maize, which  
3 stands at 26.5 tons CO<sub>2</sub> ha<sup>-1</sup> year<sup>-1</sup> [6].

4 In nature, the inorganic carbon (C) source for planktonic photosynthesis comes from the air which contains  
5 only 0.04% CO<sub>2</sub> [7]. The low dissolved CO<sub>2</sub> (dCO<sub>2</sub>) concentration in water at equilibrium (the diffusion  
6 coefficient is 10<sup>4</sup> times smaller than in air) generally cannot meet the optimal growth rate and high biomass  
7 yield [8–11]. To avoid C limitation, microalgae cultures can be injected with CO<sub>2</sub>-enriched air/flue gases  
8 [12,13]. The CO<sub>2</sub>-enriched gas injected into the photobioreactors promotes algal growth and biomass  
9 production, which is largely due to the enhanced photosynthetic performance with an increase of C  
10 availability [14,15]. The growth rate and CO<sub>2</sub>-fixation efficiency of *Chlorella vulgaris* increased by 120–  
11 150% or 6–12 times, respectively, at 10% CO<sub>2</sub> (v/v) [16] although CO<sub>2</sub> becomes toxic to this organism  
12 above the concentration of 20% (v/v) in the gas phase [17]. The C availability to cells is dependent on the  
13 feeding modes [18]. As the most affordable C source, CO<sub>2</sub> is usually supplied in the gas phase but the  
14 insufficient CO<sub>2</sub> mass transfer rate in water may cause a considerable proportion of the gas-phase CO<sub>2</sub> to  
15 escape from the culture medium, reducing its utilization efficiency [11,19]. Therefore, the improvement of  
16 CO<sub>2</sub> fixation efficiency has become a bottleneck in microalgae-based CO<sub>2</sub> capture technology.

17 In batch cultures, all the nutrients are present at the beginning of cultures and are used during growth while  
18 gaseous nutrients, such as CO<sub>2</sub> or O<sub>2</sub>, and light (in the case of photosynthetic cultures) are supplied  
19 continuously during the culture period. The growth rate is, therefore, rapidly controlled by the transfer rate  
20 of the required gases or the rate of light arrival in the ever-densifying photosynthetic cultures. These  
21 phenomena explain the carbon-limitation and/or light limitation of microalgae cultures, and the resulting  
22 linear growth phases and low final densities [20,21].

1 Environmental conditions in the photobioreactor like pH, carbonate chemistry, light and nutrients  
2 availabilities to cells alter with the increase in biomass density [22]. These may trigger changes in algal  
3 metabolism and physiology, which in turn affect biomass productivity [23,24]. The increasing pH caused  
4 by the consumption of  $dCO_2$  and bicarbonate ( $HCO_3^-$ ) through photosynthesis has been thoroughly  
5 explained by Binaghi et al. [25] and Gao [26]. Researchers also found that using  $NO_3^-$  as a nitrogen (N)  
6 source may cause  $OH^-$  release, rising pH [27,28]. Notably, if pH exceeds the optimal threshold for algal  
7 development, the intercellular acid-base homeostasis may be disrupted, preventing high biomass densities  
8 and productivities. This has been reported for many microalgae such as *C. vulgaris* [29], *Chlorella*  
9 *sorokiniana* [30] and *Chlamydomonas reinhardtii* [31]. To maximize biomass productivity, pH modulation  
10 by the injection of acids, buffers and  $CO_2$  may make sense, but these approaches lack economic feasibility  
11 due to cost and low  $CO_2$  consumption efficiency [19]. Additionally, using acids and buffers may introduce  
12 new ions affecting the salinity and the microalgae growth in some cases.

13 According to Brune and Novak [32], the dissociated proton concentrated due to  $CO_2$  diffusion mitigates pH  
14 increase. Therefore, when nitrate ( $NO_3^-$ ) is used as a sole N source, there may be a chance to cost-effectively  
15 control pH by adding  $dCO_2$ -enriched media (eCM), in other words, where  $CO_2$  is pre-dissolved into the  
16 growth medium. This process may enhance the  $CO_2$  utilization efficiency through reducing the escaping  
17 fraction, and simultaneously mitigate the pH increases caused by photosynthesis and nitrate consumption,  
18 consequently stimulating the growth. To the best of the authors' knowledge, this approach has received little  
19 consideration. Therefore, to assess the feasibility of this technique, 3N-Bristol medium (in which  $NO_3^-$  is  
20 the only N source) pre-mixed with  $CO_2$  was intermittently pumped into *C. vulgaris* cultures (fed-batch) to  
21 maintain pH, and the growth rate, biomass productivity and nutrients removal capacity of *C. vulgaris* were  
22 investigated to compare with those obtained in batch culture. Furthermore, the optimal  $CO_2$  concentration  
23 in the feed medium was determined. This study may provide an economic  $CO_2$ -feeding strategy to optimize  
24 microalgae production systems.

1

## 2    **2.       Materials and Methods**

3

### 4    *2.1. Microalgae species and culture maintenance*

5

6    *Chlorella vulgaris* CCAP211/e 11B (CCAP, Göttingen) was maintained in Erlenmeyer flasks (250 mL,  
7    containing 100 mL 3N-Bristol medium) with a weekly sub-culturing frequency. The medium contains (per  
8    litter): 748 mg NaNO<sub>3</sub>, 28 mg CaCl<sub>2</sub>·2H<sub>2</sub>O, 75 mg MgSO<sub>4</sub>·7H<sub>2</sub>O, 21 mg FeEDTA, 75 mg K<sub>2</sub>HPO<sub>4</sub>, 174 mg  
9    KH<sub>2</sub>PO<sub>4</sub>, 20 mg NaCl, 3 mg H<sub>3</sub>BO<sub>3</sub>, 6 mg MnCl<sub>2</sub>·4H<sub>2</sub>O, 303 µg ZnSO<sub>4</sub>·7H<sub>2</sub>O, 121 µg CuSO<sub>4</sub>·7H<sub>2</sub>O, 22 µg  
10   MoO<sub>3</sub> (85%, w/w), and 137 µg CoSO<sub>4</sub>·7H<sub>2</sub>O [33]. The flasks were illuminated continuously under a light  
11   intensity of 20 µmol photons m<sup>-2</sup> s<sup>-1</sup> at the surface of the cultures with a mixing (150 rpm) at 25°C in  
12   an orbital shaker incubator (Multitron, Infors HT, Switzerland).

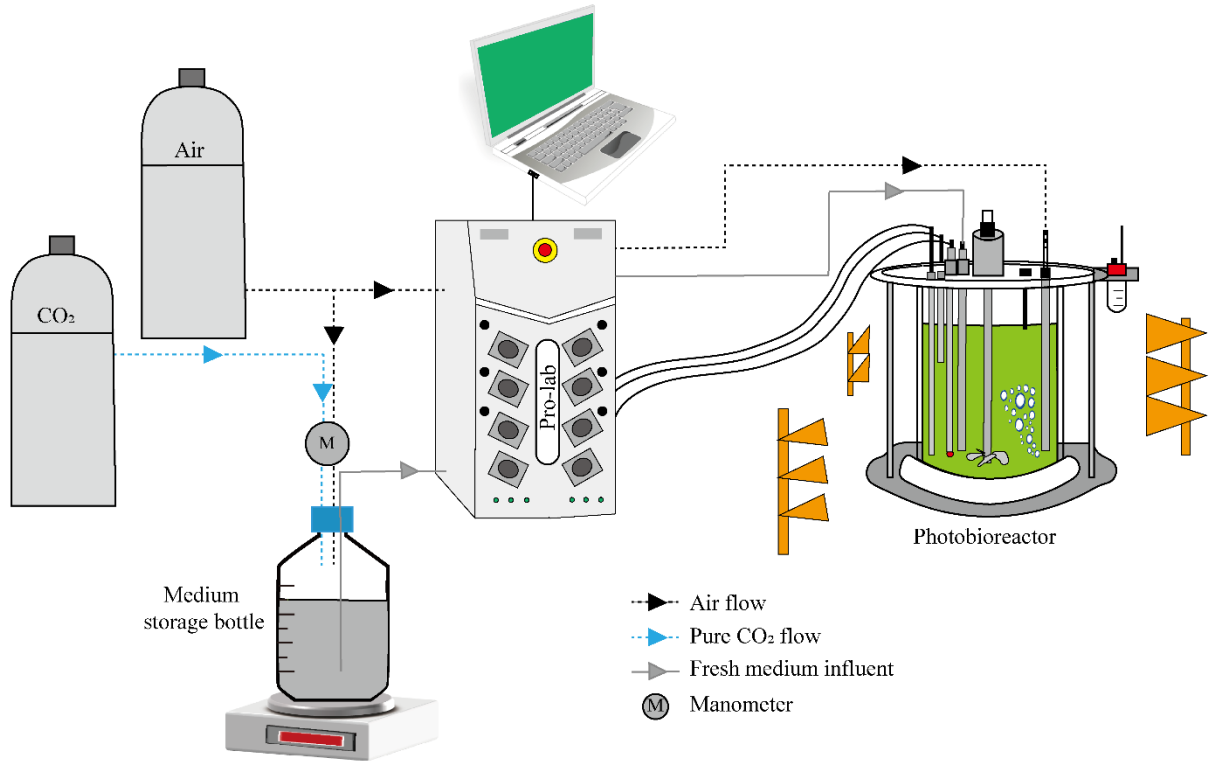
13

### 14   *2.2. Experimental procedure*

15

16   A 6.8 L baffled photobioreactor (GPC bio, France) equipped with 4 pitch-blade impellers (500 rpm) was  
17   used to grow *C. vulgaris* in batch (B) or fed-batch (FB) cultures (Fig. 1). The pH probe (EasyFerm Bio Arc  
18   325, Hamilton, Swiss) was calibrated before sterilization (121°C, 20 min) using the standard pH buffers at  
19   7.00 and 10.01 (Thermo Fisher Scientific Inc.); and the dissolved oxygen probe (VisiFerm DO Arc 325,  
20   Hamilton, Swiss) was calibrated after sterilization at 0% and 100% air-saturation of sterilized growth  
21   medium. Three LED lamps (Ledare 130, 78 lumen, 2700 Kelvin, 27° dispersion angle, IKEA, France) were  
22   used to illuminate the cultures from three directions (120° angles to each other) and the total internal light  
23   intensity was 185 µmol photons m<sup>-2</sup> s<sup>-1</sup> (QSL-2100, Biospherical Instruments, San Diego, CA, USA) at the

1 internal surface of the glass culture vessel. Temperature-control was by means of a double envelope through  
2 which water at 25 °C circulated.



3  
4 **Fig. 1.** Experimental set-up for *C. vulgaris* batch and fed-batch cultures.

5  
6 *2.2.1. Batch (B) culture*

7 The “standard” batch culture (5 L, Table 1) was inoculated with  $2.1 \times 10^{10}$  cells ( $4.2 \times 10^6$  cells mL<sup>-1</sup>). The  
8 algal suspension from section 2.1. was centrifuged at 5000 rpm for 5 min and then re-suspended with  
9 sterilized 3N-Bristol medium. The filtrated air (0.2 μm, Sartorius, Germany) was bubbled into the  
10 photobioreactor at 500 mL min<sup>-1</sup> as measured by a mass flow controller (Bronkhorst). During the  
11 experiment (8 days), no extra medium was added to the culture.

12

1 Table 1. Detailed CO<sub>2</sub>/medium feeding regimes in different cultures.

Culture mode	Culture label	pCO <sub>2</sub> in supplied medium (bar)	Culture aeration (mL min <sup>-1</sup> )	*Calculated dCO <sub>2</sub> in growth medium (g L <sup>-1</sup> )	pH control	Initial volume (L)	Final volume (L)
Batch	Batch	Air-equilibrated	500	NA	–	5	5
Fed-batch	FB_blank	Air-equilibrated	0	0.0006	+	5	5
	FB_control	Air-equilibrated	500	NA	+	0.5	5.5
	FB_1.55	1.15	0	1.55	+	0.5	5.5
	FB_1.62	1.20	0	1.62	+	0.5	5.5
	FB_1.75	1.30	0	1.75	+	0.5	5.5
	FB_1.88	1.40	0	1.88	+	0.5	5.5

2 \* represents the calculated dCO<sub>2</sub> using Henry’s law, where the constant for CO<sub>2</sub> in water at 25°C is 3.1 ×  
3 10<sup>-2</sup> M atm<sup>-1</sup>; “+” represents the culture was under pH-controlled, and “–” indicates the culture had no pH-  
4 control; “FB” stands for the fed-batch culture, FB\_control and FB\_blank respectively represent the fed-  
5 batch cultures injected with and without air in the photobioreactor, whereas FB\_1.55, FB\_1.62, FB\_1.75  
6 and FB\_1.88 are the fed-batch cultures fed with the media enriched with 1.55, 1.62, 1.75 and 1.88 g L<sup>-1</sup>  
7 dCO<sub>2</sub>, respectively; NA, Not applicable.

8

### 9 2.2.2. Fed-batch (FB) culture

10 For the FB cultures, the bioreactor was initially inoculated with a population of  $4.3 \pm 2.8 \times 10^9$  cells  
11 (approximately 5 times less than that of the batch culture) in 0.5 L medium, the starting volume of the FB  
12 cultures. A 10 L bottle containing 5 L 3N-Bristol medium was connected to the bioreactor through the acid-  
13 feeding pump (Fig. 1). The medium in the bottle was previously adjusted to five different absolute pCO<sub>2</sub>  
14 levels (Table 1): atmospheric (blank and control), and pCO<sub>2</sub>-enriched media (eCM) at 1.15, 1.20, 1.30 and  
15 1.40 bar which respectively corresponds to the dCO<sub>2</sub> concentrations of 1.55, 1.62, 1.75 and 1.88 g L<sup>-1</sup>



1 (Henry's law). For each target dCO<sub>2</sub>-enriched concentration, the growth medium was allowed to reach  
2 equilibrium with a different pressure of pure CO<sub>2</sub> gas that was bubbled into the medium. The pressure was  
3 maintained at the desired level for the duration of the experiment by means of a permanent connection to a  
4 CO<sub>2</sub> gas cylinder. In each experiment, after inoculation, the medium was automatically fed into the  
5 bioreactor according to the command of the controller to maintain the culture-pH at 6.5 until the maximal  
6 volume (5.5 L) was attained. There was no aeration of the culture for neither the FB 'blank' experiment nor  
7 for any of the FB dCO<sub>2</sub>-enriched cultures.

8

### 9 2.3. *Analysis*

10

#### 11 2.3.1. *Total population*

12 The cell number was determined by particle counting and the associated characterization system (Beckman  
13 Coulter Multisizer 4e counter, Beckman, US). Sample preparation involved taking 100 μL of sample with  
14 the appropriate dilution (in a total volume of 10 mL). Measurements were performed in triplicates from each  
15 sample. Total population (P, cells) in the photobioreactor was calculated by the equation:

$$16 \quad P = C \times V \quad (1)$$

17 Where V is the volume (L) of algal culture in the photobioreactor at the time of sampling and C is the cell  
18 concentration (cells L<sup>-1</sup>) of the culture at the same time.

19

#### 20 2.3.2. *Growth rate and biomass productivity*

21 The specific growth rate (μ, h<sup>-1</sup>) of cultures was estimated using the equation [34]:

$$22 \quad \mu = [\ln(P_t) - \ln(P_{t_0})] / (t - t_0) \quad (2)$$

1 Where  $P_{t_0}$  and  $P_t$  are the total populations at the times  $t$  and  $t_0$  (h), respectively. The maximal specific growth  
2 rate ( $\mu_{\max}$ ,  $\text{h}^{-1}$ ) was calculated during the exponential phase and the doubling time ( $T_d$ , h) was calculated  
3 from the maximal specific growth rate by the equation [35]:

4 
$$T_d = \ln(2) / \mu_{\max} \quad (3)$$

5 The biomass concentration ( $X$ ,  $\text{mg L}^{-1}$ ) was quantified by dry weight (DW) measurements, which was  
6 carried out by a centrifugation of approximately 50 mL sample, followed by a washing protocol using  
7 deionised  $\text{H}_2\text{O}$  and transfer to a pre-weighed ceramic crucible. The ceramic crucible was then placed at  
8  $105^\circ\text{C}$  for 5 days and re-weighed as soon as possible after cooling. The biomass productivity ( $P_x$ ,  $\text{mg L}^{-1} \text{d}^{-1}$ )  
9 was calculated by the equation:

10 
$$P_x = (X \cdot V - X_0 \cdot V_0) / t \quad (4)$$

11 Where  $X_0$  and  $V_0$  refer to the biomass concentration and culture volume in the bioreactor at the beginning  
12 of the experiments, respectively; and  $t$  is the cultivation period (days). Also, the maximum biomass  
13 productivity ( $P_{\max}$ ) of cultures under different conditions was calculated depending on the biomass changes  
14 during the exponential phase.

15

### 16 2.3.3. Nitrogen/Phosphate removal capacity

17 In order to assess the nutrients (mainly in terms of nitrogen, N, and phosphorus, P) removal capacity  
18 of *C. vulgaris* under different  $\text{dCO}_2$  levels, the concentrations of  $\text{N-NO}_3^-$  and  $\text{P-PO}_4^{3-}$  in algal  
19 solution were determined using an Ion Chromatography (ICS-5000, ThermoFisher Scientific Inc,  
20 USA) at the beginning and end of the experiments. All the samples were filtrated with  $0.2 \mu\text{m}$   
21 filters and stored at  $-20^\circ\text{C}$  before analysis. The total nutrients in feed-batch cultures involved two  
22 parts:  $\text{N-NO}_3^-$  and  $\text{P-PO}_4^{3-}$  in the bioreactor and those supplied from the medium storage bottle.  
23 Therefore, the nutrients uptake rate ( $N_{\text{uptake}}$ ,  $\text{mg d}^{-1} \text{L}^{-1}$ ) was calculated by the equation:

1 
$$N_{uptake} = [C_0 (V_0 + V_{add}) - C_{end} V_{end}] / t \quad (5)$$

2 Where  $C_0$  and  $C_{end}$  respectively represent the  $N-NO_3^-$  or  $P-PO_4^{3-}$  concentration ( $mg L^{-1}$ ) in the  
3 bioreactor at the beginning and end of cultivation;  $V_0$  and  $V_{end}$  respectively represent the culture  
4 volume in the bioreactor at the beginning and end of cultivation, and  $V_{add}$  is the volume of medium  
5 that was pumped into the bioreactor (where  $V_{add}$  is 0 for the batch culture).

6

#### 7 2.3.4. pH control

8 The pH of *C. vulgaris* cultures was measured continuously. For the batch culture, the pH was allowed to  
9 change freely. The growth medium-feed for the FB cultures, on the other hand, was linked to the variations  
10 in pH. Due to the volume limitation of photobioreactor, the FB growth could be divided into phases: a fed-  
11 batch phase (FB-phase), during which the growth medium was added to keep pH constant (6.5 ~ 6.8) [36];  
12 and a batch phase (B-phase), after the addition of all the growth medium (5 L), during which these cultures  
13 entered the batch culture mode and the pH was allowed to fluctuate freely.

14

### 15 3. Results

16

17 The experiments were designed to compare  $CO_2$  utilization efficiency when  $CO_2$  was pre-dissolved in the  
18 growth medium with when it was supplied continuously in gaseous form (aeration in the culture vessel). A  
19 batch experiment was performed under the “standard” condition. This is comparable to the FB\_control  
20 experiment where the medium was fed into an air-aerated bioreactor. In the FB\_blank experiment, the  
21 growth medium was in equilibrium with the air and fed into the culture using pH control. No aeration was  
22 applied in the culture vessel under this experimental setting. The FB d $CO_2$ -enriched experiments used media  
23 in equilibrium with pure  $CO_2$  gas at different absolute pressures, achieving a series of  $CO_2$  concentrations

1 dissolved into the growth media (Table 1). The feeding regime for all FB cultures was based on the addition  
2 of growth media through the acid pump to keep the culture pH stable.

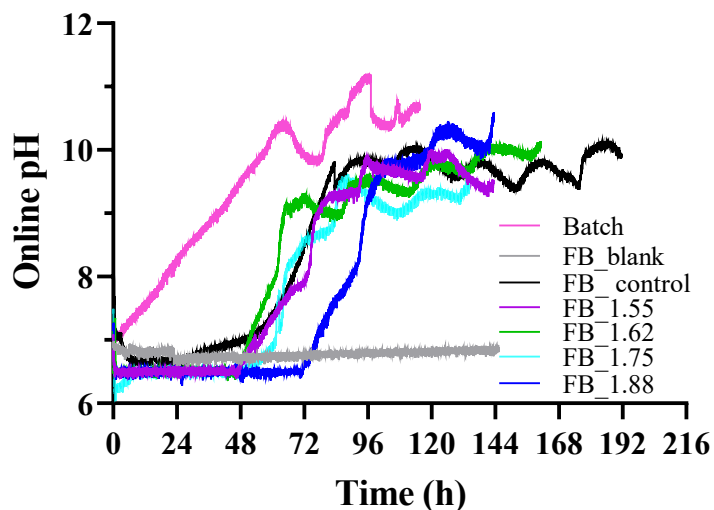
3

### 4 3.1 pH changes during growth

5

6 In the “standard” batch culture, the pH progressively increased in the first 48h, and then fluctuated between  
7 10.0 and 11.2. In the FB\_blank culture, the pH was controlled and remained constant at approximately 6.8.  
8 For the other FB cultures, their pH values remained constant at 6.5 (controlling point) while they were being  
9 fed with eCMs (at FB-phase). Once the growth media were all added into the culture vessel, the cultures  
10 passed into batch mode (at B-phase) and the pH increased sharply and finally plateaued around 9.8.

11



12

13 **Fig. 2.** pH variations of *C. vulgaris* cultures under different feeding regimes (“FB” stands for the fed-batch  
14 culture, FB\_control and FB\_blank respectively represent the fed-batch cultures injected with and without  
15 air in the photobioreactor, whereas FB\_1.55, FB\_1.62, FB\_1.75 and FB\_1.88 are the fed-batch cultures fed

1 with the media enriched with 1.55, 1.62, 1.75 and 1.88 g L<sup>-1</sup> dCO<sub>2</sub>, respectively. Same legends also apply  
2 to the following figures and tables).

3

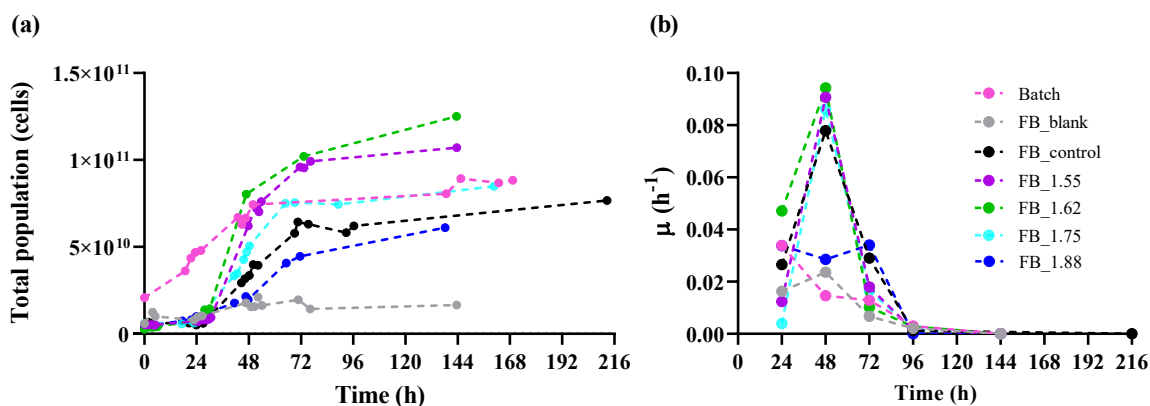
### 4 3.2. Growth and productivity of *C. vulgaris* under different feeding regimes

5

6 In batch culture, the growth was linear from the beginning until 48h, yielding a final population of  $8.06 \times$   
7  $10^{10}$  cells and 1140 mg DW at the end (Fig. 3a, Table 2). Considering that the growth stopped at 48h, a  
8 maximum biomass productivity ( $P_{\max}$ ) of 79.9 mg DW L<sup>-1</sup> d<sup>-1</sup> was obtained (Table 2).

9 All FB cultures presented a classical growth pattern consisting of the lag, exponential and stationary phase  
10 (Fig. 3a). The FB\_control culture achieved a final population of  $7.67 \times 10^{10}$  cells (or 1441 mg DW, Table 2),  
11 which is comparable to the final biomass of batch culture under the “standard” condition. The final *C.*  
12 *vulgaris* biomass produced in FB cultures was related to the dCO<sub>2</sub> concentrations in the feeding media. The  
13 culture fed with growth medium with a dCO<sub>2</sub> concentration at 1.62 g L<sup>-1</sup> exhibited the highest final  
14 population ( $1.3 \times 10^{11}$  cells, Fig. 3a), 1.6 times higher than that of the batch and FB\_control cultures. The  
15 FB cultures with lower or higher dCO<sub>2</sub> concentrations had lower final populations.

16



17

1 **Fig. 3.** Total population (a) and specific growth rate (b) of *C. vulgaris* in different cultures (same legends  
 2 in b also apply to a).

3  
 4 In batch culture, the growth rate ( $\mu$ ) decreased during the culture period with a maximum value of  $0.034 \text{ h}^{-1}$   
 5  $^{-1}$  determined during the first 24h (Fig. 3b, Table 2). This was 2.3 times lower than the  $\mu_{\text{max}}$  ( $0.078 \text{ h}^{-1}$ )  
 6 estimated in the FB\_control culture. The growth rates of all FB cultures greatly increased on the second day  
 7 of culture and then decreased progressively, except for the FB culture at  $1.88 \text{ g L}^{-1} \text{ dCO}_2$ , with a constant  $\mu$   
 8 at around  $0.032 \text{ h}^{-1}$  during the first 72 hours. The culture grown at  $1.62 \text{ g L}^{-1} \text{ dCO}_2$  exhibited the highest  
 9 growth rate ( $\mu_{\text{max}} = 0.094 \text{ h}^{-1}$ ), closely followed by the  $\mu_{\text{max}}$  for the cultures at  $1.55$  and  $1.75 \text{ g L}^{-1}$ . After 72h,  
 10 near-zero growth rates for all cultures were observed (Fig. 3 b).

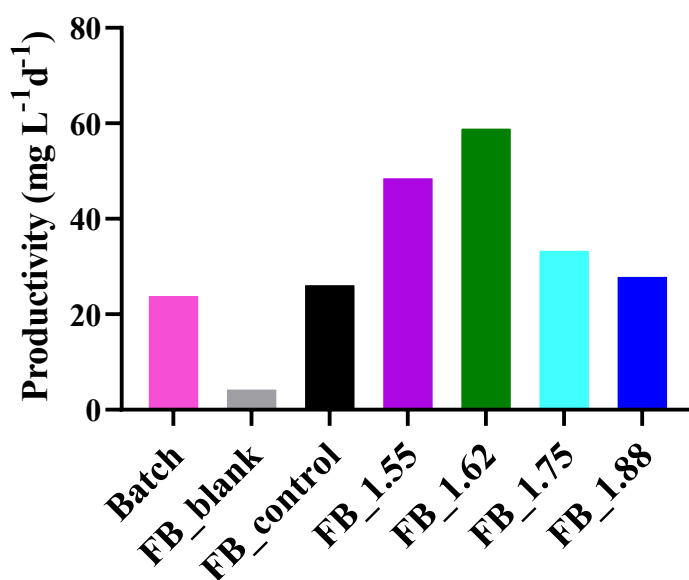
11  
 12 Table 2. Growth parameters (maximum specific growth rate, doubling time, maximum biomass  
 13 productivity and final biomass) of *C. vulgaris* cultures under different feeding regimes.

Treatment	Duration of experiments (h)	Maximum specific growth rate ( $\mu_{\text{max}}$ , $\text{h}^{-1}$ )	$R^2$ for $\mu_{\text{max}}$	Doubling time ( $T_d$ , h)	Maximum productivity ( $\text{mg L}^{-1} \text{ d}^{-1}$ )	Final biomass production (mg)
Batch	169	0.034	0.90	20.4	79.9	1140
FB_blank	144	0.024	0.95	28.9	17.2	264
FB_control	212	0.078	0.97	8.9	86.5	1441
FB_1.55	144	0.091	0.98	7.6	168.2	1744
FB_1.62	139	0.094	1.00	7.4	222.3	2040
FB_1.75	161	0.086	0.99	8.1	123.0	1381
FB_1.88	138	0.034	0.98	20.4	40.8	996

14

1 Without continuous air-injection, the FB\_blank culture showed the lowest final biomass productivity of 4.5  
2  $\text{mg L}^{-1} \text{d}^{-1}$  (Fig. 4) while the batch and FB\_control cultures at atmospheric  $\text{dCO}_2$  concentrations achieved a  
3 higher productivity around  $25.0 \text{ mg L}^{-1} \text{d}^{-1}$ . Also, the final biomass productivity in the FB cultures peaked  
4 ( $59.5 \text{ mg L}^{-1} \text{d}^{-1}$ ) at a  $\text{dCO}_2$  concentration of  $1.62 \text{ g L}^{-1}$ . The  $\text{dCO}_2$  dosage-dependent effect on the  $P_{\text{max}}$  of *C.*  
5 *vilgaris* cultures was consistent with that on their final biomass productivities (Fig. 4, Table 2).

6



7

8 **Fig. 4.** Biomass productivity of *C. vilgaris* cultures grown under different feeding regimes.

9

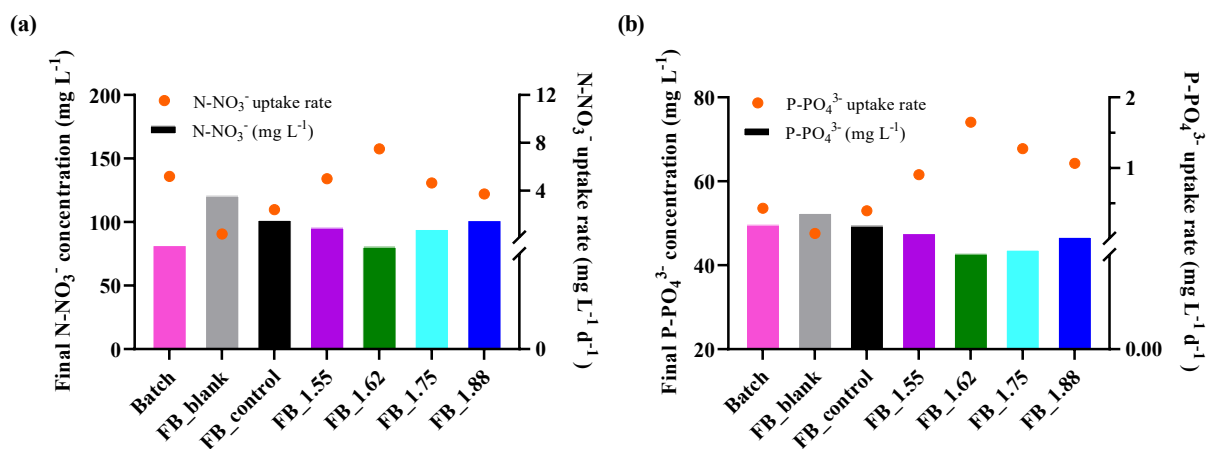
#### 10 3.4. Effect of $\text{dCO}_2$ on *C. vilgaris* nutrients uptake

11

12 In the FB cultures, the residual  $\text{N-NO}_3^-$  and  $\text{P-PO}_4^{3-}$  concentrations at the end of the culture period decreased  
13 with the increasing medium  $\text{dCO}_2$  up to the value of  $1.62 \text{ g L}^{-1}$  and then increased with the higher  $\text{dCO}_2$   
14 concentrations (Fig. 5). Conversely, the nutrients uptake rates were stimulated by the increasing  $\text{pCO}_2$  and

1 then lowered. The maximum uptake rates of N-NO<sub>3</sub><sup>-</sup> and P-PO<sub>4</sub><sup>3-</sup> (7.5 and 1.6 mg L<sup>-1</sup> d<sup>-1</sup>, respectively) were  
 2 both observed in the FB\_1.62 g L<sup>-1</sup> dCO<sub>2</sub> culture. On the other hand, the FB\_blank culture displayed the  
 3 lowest nutrients uptake rates. The batch culture showed higher N-NO<sub>3</sub><sup>-</sup> (2.2 times) and P-PO<sub>4</sub><sup>3-</sup> (1.1 times)  
 4 uptake rates than those for the FB\_control culture, but significantly lower (0.25 and 0.69 times, respectively)  
 5 than those for FB\_1.62 g L<sup>-1</sup> dCO<sub>2</sub> culture.

6



7

8 **Fig. 5.** Final nutrients (N-NO<sub>3</sub><sup>-</sup>, a; and P-PO<sub>4</sub><sup>3-</sup>, b) concentrations and uptake rates of *C. vulgaris* under  
 9 different feeding regimes.

10

## 11 4. Discussion

12

### 13 4.1. Effect of feeding modes on the growth of *C. vulgaris*

14

15 The pH-controlled FB operational mode proved a successful method for controlling and piloting cultures of  
 16 *C. vulgaris*. Under atmospheric dCO<sub>2</sub> conditions, FB culture grew faster than B culture, although the final  
 17 biomass concentration was not any higher (Fig. 2, Table 2). This suggests that the FB feeding mode may



1 outperform B culture for microalgae production. Similar findings have been reported in green algae  
2 *Scenedesmus sp.* and cyanobacteria such as *Microcystis ichthyoblabe* and *Spirulina platensis* [25,37,38].  
3 Wang et al. [13] also reported that under high illumination, the FB culture of *Nannochloropsis oculata*  
4 boosted the algal growth as this feeding mode improved algal tolerance to adverse conditions. In our study,  
5 the fed-batch culture mode stimulated the algal growth probably by alleviating the negative effects of  
6 undesired parameters, such as a greater CO<sub>2</sub> limitation and light attenuation, which may occur in the batch  
7 culture.

8 In order to avoid a growth inhibition by the development of unfavourable factors (e.g. high pH, nutrients  
9 starvation and auto-shading effects) at the beginning of the experiments, the FB cultures were inoculated  
10 with a fifth of the population than the batch culture (with a small volume of medium, 0.5 L). Indeed, the  
11 growth conditions/environmental parameters are highly dependent on the biomass density [26]. The linear  
12 growth in the batch culture within the first 48h (Fig. 2) strongly suggests that the algal growth was factor(s)-  
13 limited [21]. During this period, the rise of pH in the batch culture (Fig. 2) may suggest declining dCO<sub>2</sub>  
14 concentration in the presence of an increasing population as a limiting factor. This might be because the  
15 photosynthetic CO<sub>2</sub> removal rate exceeded that of the CO<sub>2</sub> transfer rate from air to liquid [12,26]. The pH  
16 of the FB\_control culture remained constant for longer than in the batch culture. This could be due to the  
17 intermittent addition of growth medium at a lower pH into the culture brought about by the FB mode.  
18 Additionally, although the bioprocess of nitrogen assimilation releases OH<sup>-</sup>, which normally increases pH,  
19 no nutrients limitation for growth was observed in both cultures (Fig. 5).

20 During the FB-phase, the volume of FB cultures was lower than that of batch culture. One can imagine that  
21 the light availability for the FB cultures was greater due to a lower population and volume of liquid [39].  
22 Thus, the photons (energy) accessible to each cell in the bioreactor were likely to be lower in the B culture.  
23 This could be another reason for the lower biomass productivity when using the batch culture mode. In order  
24 to better understand the influence of feeding mode on the light profiles and the subsequent effect on biomass

1 accumulation, the availability of light to individual cells in both cultivation modes should be estimated in  
2 future studies.

3

#### 4 4.2. Effect of $dCO_2$ concentration on the growth and biomass productivity of *C. vulgaris*

5

6 When  $CO_2$  is used as the sole inorganic carbon source for autophototrophic cultures, two factors influencing  
7 algal growth should be considered: 1) carbon availability to cells, and 2) culture pH [40,41]. Before reaching  
8 the target volume, all the FB cultures remained at the optimal pH at 6.5 though there was a slight increase  
9 in the pH of the FB\_control culture (Fig. 2). This was probably because the feed medium in this culture was  
10 not acidified enough.

11 The differences in growth performances of FB cultures during the FB-phase could be attributed to the  
12 different carbon availabilities in the growth media. The overall growth profile of *C. vulgaris* under various  
13 conditions suggests that algal growth was C-limited at low  $dCO_2$  concentrations and inhibited at high  $dCO_2$   
14 concentrations. This finding is in line with previous studies investigating the impact of  $CO_2$  concentrations  
15 on the development of microalgae [10,13,42]. In this study, one may classify the *C. vulgaris* cultures into  
16 four groups according to their growth profiles with respect to the  $CO_2$  supplement regimes: strongly C-  
17 limited group (FB\_blank culture), slightly C-limited group (batch and FB\_control cultures), C-saturated  
18 group (FB\_1.55 and FB\_1.62  $g L^{-1}$  cultures) and C-toxic group (FB\_1.75 and 1.88  $g L^{-1}$  cultures).

19 In the FB\_blank culture, the rapid consumption of  $CO_2$  by the algae probably resulted in a C-deficient  
20 environment that suppressed algal growth. The  $CO_2$  captured through photosynthesis makes up the C-  
21 skeleton of macromolecules, such as enzymes, nucleic acids and polysaccharides, that are requisites for  
22 algal metabolisms, growth and reproduction [43]. Therefore, the lowest growth and population increase of  
23 *C. vulgaris* in this  $CO_2$  feeding regime was likely hampered by a constraint in photosynthetic substrate ( $CO_2$ )  
24 availability rather than an altered pH. Compared to the FB\_blank culture, the growth rate and biomass

1 productivity for the batch and FB\_control cultures were higher, which could be related to higher CO<sub>2</sub>  
2 availability in these cultures due to continuous air injection. Despite the greater availability of CO<sub>2</sub> in these  
3 two cultures, C limitation was still observed, as indicated by the increasing specific growth rate when *C.*  
4 *vulgaris* was grown in 1.55 and 1.62 g L<sup>-1</sup> of dCO<sub>2</sub> ( $\mu_{\max}$  of 0.091 and 0.094 h<sup>-1</sup>, respectively). Limited  
5 growth under atmospheric conditions has also been reported in the other *Chlorella* strains [17,44,45], and  
6 other microalgae such as *N. oculata* [13] and *Scenedesmus obliquus* [10].

7 A moderate increase in the availability of CO<sub>2</sub> promotes algal growth [10,17]. In this study, the better media  
8 for *C. vulgaris* growth contained 1.55 and 1.62 g L<sup>-1</sup> dCO<sub>2</sub> where the cultures achieved the highest growth  
9 rates, final biomass productions and productivities, as well as the shortest doubling time (Table 2).  
10 Increasing dCO<sub>2</sub> further (i.e., 1.75 and 1.88 g L<sup>-1</sup>) reduced the growth rate, indicating that excess CO<sub>2</sub>  
11 supplements may be inhibitory to *C. vulgaris*. Whitney et al. [46] has illustrated that high CO<sub>2</sub> concentration  
12 decreased the transmission of photoelectron signals, affecting algal photosynthetic activity and thereby  
13 lowering the biomass productivity. High CO<sub>2</sub> concentration has also been found to induce reactive oxygen  
14 species and oxidative stress, inhibiting the growth of *Arthrospira platensis* [47] and *S. obliquus* [10]. Despite  
15 the CO<sub>2</sub> inhibition at higher levels, the FB\_1.75 g L<sup>-1</sup> culture still had superior growth parameters than those  
16 for the FB\_control culture. This could be due to that the CO<sub>2</sub> toxicity was offset by the increased carbon  
17 availability.

18

#### 19 4.3. Effect of pCO<sub>2</sub> level on the nutrients uptake capability of *C. vulgaris*

20

21 In the FB cultures, the nutrients (N and P) uptake trends followed those of growth rate and biomass  
22 productivity, and peaked at a dCO<sub>2</sub> concentration of 1.62 g L<sup>-1</sup>. This indicates that a moderately CO<sub>2</sub>-  
23 enriched medium could improve the nutrients removal capacity of microalgae. The data is in agreement  
24 with Almomani et al. [8] who have discovered that with the increase of CO<sub>2</sub> concentration, the N and P

1 removal efficiencies of *S. platensis* and multispecies-microalgae cultures increased at first (< 10% CO<sub>2</sub>, v/v)  
2 and then decreased with higher CO<sub>2</sub> concentrations (> 15%). Likewise, Hu et al. [44] also found that  
3 *Chlorella sp.* showed low N removal rates (87-89%) under atmospheric and 10% CO<sub>2</sub> conditions, with the  
4 greatest rate (96%) at 5% CO<sub>2</sub>. CO<sub>2</sub> concentration may modulate the efficiency of photosynthesis, which  
5 fuels cellular metabolisms including nutrients absorption and assimilation [48]. It appears that the optimal  
6 CO<sub>2</sub> concentration could accelerate photosynthetic activity and then, indirectly enhance the nutrients uptake  
7 traits. Notably, *C. vulgaris* grown in batch culture presented higher N/P uptake rates than those for the  
8 FB\_control culture, which could be related to a higher nutrients consumption due to the larger population  
9 at the beginning.

10

#### 11 4.4. Feeding CO<sub>2</sub>-enriched growth media may enhance the greenhouse gas capture by *C. vulgaris*

12

13 The pH in the FB cultures was maintained at 6.5 during the FB-phase. Under these conditions, the  
14 predominant inorganic carbon sources are free CO<sub>2</sub> (aq) and HCO<sub>3</sub><sup>-</sup> in equilibrium [32], both of which are  
15 the species readily used by photosynthesis. So maintaining this pH (at 25°C) means that over 99% of the  
16 carbon species is available to the microalgae [32,49]. The main advantage of pre-dissolving the CO<sub>2</sub> into  
17 the feed medium over running a batch culture which has pH control with CO<sub>2</sub> injection is that in the former  
18 case, there are no CO<sub>2</sub> losses from the bioreactor. One may therefore suspect a high CO<sub>2</sub> utilization  
19 efficiency by pre-dissolving CO<sub>2</sub> into the growth medium, compared to the bubbling systems where a large  
20 fraction of CO<sub>2</sub> escapes in gas form [19,50]. A high CO<sub>2</sub> removal rate of up to 96% in *C. vulgaris* culture  
21 (in batch) within 1h was determined after CO<sub>2</sub> was pre-dissolved in the growth medium [51].

22 The CO<sub>2</sub> capture efficiency of *C. vulgaris* was estimated in gas-injecting bioreactors up to 80% [24] (Table  
23 3). To enhance CO<sub>2</sub> bioremediation and mitigate the greenhouse effects, optimization procedures such as  
24 improving photobioreactors, gas flow rate, and algal acclimatization to high CO<sub>2</sub> concentrations or species

1 screening are required (Table 3) [24,50]. This study may provide a new approach for effectively capturing  
 2 CO<sub>2</sub> through feeding CO<sub>2</sub>-enriched media (even with high dCO<sub>2</sub>). An additional benefit would be in the  
 3 startup phase of larger configurations where normally a large volume of starter culture is required.  
 4 Dissolving the CO<sub>2</sub> in the feed medium also simplifies the optimization of bioprocesses.

5

6 Table 3. CO<sub>2</sub> utilization efficiency of *Chlorella* species in gas-injecting photobioreactors.

Species	Bioreactor	Enriched CO <sub>2</sub> concentration	Aeration rate	CO <sub>2</sub> removal efficiency	Reference
<i>Chlorella vulgaris</i>	Bubble column photobioreactors	4*, 12%	1*, 2, 5×10 <sup>-3</sup> m s <sup>-1</sup>	3.8-14.6%	[52]
<i>Chlorella sp. L166</i>	Erlenmeyer flask	5*, 10%	0.05, 0.10*, 0.15 vvm	< 27%	[44]
<i>Chlorella sp. P12</i>	Open thin-layer photobioreactor	Flue gas (6-8%)*, Pure CO <sub>2</sub>	5*-35 m <sup>3</sup> h <sup>-1</sup>	10-50%	[53]
<i>Chlorella sp.</i>	Semicontinuous photobioreactor	2*, 5, 10, 15%	0.25 vvm	16-58%	[54]
<i>Chlorella sp. NCTU-2</i>	Air-lift photobioreactors	10%	0.125*, 0.25, 0.5 vvm	< 63%	[55]
<i>Chlorella vulgaris</i>	Air-lift photobioreactor	2%	7.5*, 13.3×10 <sup>-3</sup> m s <sup>-1</sup>	57-80%	[24]

7 \* represents the optimized conditions under which microalgae achieved the highest CO<sub>2</sub> removal  
 8 efficiency.

9

1 Flue gases, containing 3 – 30% v/v CO<sub>2</sub>, present a viable and cost-effective alternative carbon source for  
2 microalgae cultivation [56,57]. The flue-gases injected cultures for microalgae, such as *Chlorella* [58],  
3 *Chlamydomonas* [59] and *Haematococcus* [60], have been enormously documented in previous studies.  
4 Compared to those cultures, microalgae cultures feeding with media that are pre-dissolved with the waste  
5 gases might potentially achieve superior rates of CO<sub>2</sub> bio-sequestration, according to the explanation above.  
6 Nevertheless, a significant challenge lies in the implementation of this strategy for flue gases. The  
7 coexistence of inhibitory compounds, such as SO<sub>x</sub> and NO<sub>x</sub>, coupled with high temperatures, may create  
8 unfavorable conditions that impede microalgae productivity [61,62]. As a consequence of targeting  
9 enriched-dCO<sub>2</sub> concentrations by increasing pressure, the concomitant rise of toxic pollutants in the growth  
10 medium would limit biomass productivity and subsequent CO<sub>2</sub> fixation efficiency. Hence, in further studies  
11 contemplating the adoption of flue gases using the culture technique established in this work, the necessity  
12 for screening SO<sub>x</sub>/NO<sub>x</sub>/thermal-tolerant species becomes evident.

13 Overall, the results demonstrated the superiority of the pH-controlled FB culture mode over batch cultivation  
14 in biomass production and CO<sub>2</sub> biofixation. This was achieved by dissolving CO<sub>2</sub> into the feeding medium,  
15 effectively mitigating CO<sub>2</sub> limitation arising from inadequate mass transfer. Simultaneously, starting with a  
16 lower volume of the culture may help alleviate potential light limitation. In this study, an optimal  
17 concentration of dCO<sub>2</sub> was also identified for *C. vulgaris*. Maintaining cultures at optimal pH with the  
18 addition of dCO<sub>2</sub>-enriched media rather than directly injecting gaseous CO<sub>2</sub> may be an effective approach  
19 for maximizing CO<sub>2</sub> utilization by the microalgae. This operational mode could be most applicable to closed  
20 photobioreactors, particularly in pilot- or large-scale cultivation scenarios.

21

## 22 **5. Conclusions**

23

24 FB cultures with dCO<sub>2</sub>-enriched media and feeding based on maintaining a constant culture pH was a  
25 promising culture strategy for simultaneously enhancing the biomass productivity and CO<sub>2</sub> removal capacity

1 of *C. vulgaris*. By dissolving CO<sub>2</sub> into the growth medium, the losses to the atmosphere were greatly reduced  
2 and faster growth could be obtained by keeping the culture pH constant. Using this novel technique, the  
3 optimal dCO<sub>2</sub> in the medium for *C. vulgaris* growth was determined to be 1.62 g L<sup>-1</sup>. At this concentration,  
4 the  $\mu_{\max}$ , P<sub>max</sub>, maximum N and P uptake rates determined for *C. vulgaris* were 0.094 h<sup>-1</sup>, 222 mg L<sup>-1</sup> d<sup>-1</sup>, 7.5  
5 and 1.6 mg L<sup>-1</sup> d<sup>-1</sup>, respectively. Furthermore, lower growth rate was observed under conditions of low CO<sub>2</sub>-  
6 availability and high CO<sub>2</sub>-toxic. Therefore, the pH-controlled FB culture of microalgae, based on dissolution  
7 of CO<sub>2</sub> in the feed medium, provides a promising technology for effective CO<sub>2</sub> capture.

8

### 9 **Author Contributions**

10 Conceptualization, B. Taidi and C. C. Wang; Formal analysis, B. Taidi, S. F. Li and C. C. Wang; Funding  
11 acquisition, B. Taidi; Methodology, B. Taidi and C. C. Wang; Supervision, B. Taidi; Visualization, B.  
12 Taidi and C. C. Wang; Writing-original draft, review and editing, B. Taidi and S. F. Li. All authors have  
13 read and agreed to the published version of the manuscript.

14

### 15 **Data Availability Statement**

16 The raw data supporting the conclusions of this article will be made available by the authors, without  
17 undue reservation.

18

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3

#### 4 **Conflicts of Interest**

5 The authors declare no conflict of interest.

6

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